Biotransformation and Elimination of the Herbicide Dinitramine in Carp

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Carp (*Cyprinus carpio*) exposed to 1 mg/L of dinitramine (N^3,N^3-diethyl-2,4-dinitro-6-trifluoromethyl-*m*-phenylenediamine) for 12 h accumulated the herbicide. After the fish were held in fresh water for 7 days, samples of plasma, muscle, and gallbladder bile contained 6, 25, and 7%, respectively, of the concentration found immediately after exposure. Metabolites were not found in plasma or muscle, but deethylated dinitramine was present in gallbladder bile; biotransformation by dealkylation thus occurred in the liver.

Herbicides are the fastest growing type of pesticide and currently account for about half of all pesticide sales. Widespread use of herbicides may lead to introduction of these compounds into aquatic environments by runoff caused by large amounts of rainfall soon after application, by direct application over water, or by possible deliberate misuse. Capabilities of fish to cope with herbicides need to be further delineated.

Dinitramine (N^3,N^3-diethyl-2,4-dinitro-6-trifluoromethyl-*m*-phenylenediamine), the active ingredient in the herbicide cobex, is registered for use on cotton, soybeans, lima beans, peanuts, and dry beans (Berg, 1975). Metabolism of the herbicide by microorganisms was reported by Laanio et al. (1973) and Smith et al. (1973), who suggested probable pathways of degradation.

Olson et al. (1975) reported that dinitramine accumulated in carp (*Cyprinus carpio*) and channel catfish (*Ictalurus punctatus*) after a single 12-h exposure and that the residues were not eliminated during a 24-h withdrawal period in fresh water. The biotransformation of dinitramine was not investigated in the 1975 study. Therefore, we undertook the present study to better define the residue dynamics of the herbicide in fish during a longer elimination time and to investigate possible biotransformation by the fish.

MATERIALS AND METHODS

U.S. Borax and Chemical Company supplied the dinitramine (99+%) and three purified compounds that had been isolated in metabolism studies with other organisms (Table I). Pesticide grade solvents were used for all residue work, and commercial grade acetone was used to prepare the stock solution for exposure of the fish to the herbicide. The exposure was done in an area that received no sunlight nor direct artificial light.

Carp were exposed to 1 mg/L of dinitramine for 12 h at 12 °C and were held as long as 7 days in herbicide-free water. Feed was offered daily beginning on the second day after exposure. Samples of plasma, muscle, and gallbladder bile were collected from the control and five test fish after each selected sample period of 0, 1, 3, and 7 days. Plasma and bile samples were collected by syringe, extracted immediately with ether, and analyzed on a gas chromatograph (GC). Muscle samples were collected by filleting; the frozen fillets were ground, extracted, cleaned up with Florisil, and analyzed using GC (Olson et al., 1975).

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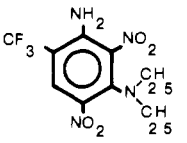
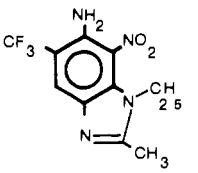
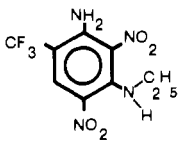
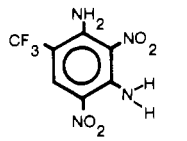
Table I. Dinitramine and Three Known Metabolites

Dinitramine	N^3,N^3-diethyl-2,4-dinitro-6-trifluoromethyl-m-phenylenediamine	
Metabolite II	6-amino-1-ethyl-2-methyl-7-nitro-5-trifluoromethylbenzimidazole	
Metabolite III	N^3-ethyl-2,4-dinitro 6-trifluoromethyl-m-phenylenediamine	
Metabolite V	2,4-dinitro-6-trifluoromethyl-m-phenylenediamine	

An improvement in the technique for analyzing muscle (fillets) gave 89.2 and 91.4% recovery from fortified samples as compared with the 70.4 and 73.1% reported by Olson et al. (1975). Evaporation of the solvents used for extraction and cleanup with a stream of cool air apparently caused less dinitramine to be lost through evaporation than was lost when samples were evaporated using a hot water bath.

Mass spectra of dinitramine and metabolite V were obtained using a Perkin-Elmer Model 270-B GC/MS with a 1 m × 2 mm glass column packed with 3% OV-7 on Chromosorb W-HP. The GC/MS system was interfaced with a PDP-12 LDP Computer. The column temperature was programmed from 100° to 240 °C at 5 °C/min with 20 psig helium as the carrier gas. Spectra scans were initiated and acquired under computed program control every 4 s with a scan duration of 4 s. Perfluorokerosine was used for calibration of the mass spectrometer.

All GC columns were 180 cm × 4 mm glass columns packed with Chromosorb W-HP and operated at 195 °C with 60 psig nitrogen. The following liquid phases were used for quantitation: 3% OV-1, dinitramine in plasma, metabolite III in bile; 5% OV-3, dinitramine in muscle, metabolite V in bile; and 3% OV-7, dinitramine in bile.

Table II. Concentration (mg/kg or mg/L) of Dinitramine and Metabolites in Carp Muscle, Plasma, and Gallbladder Bile after Bath Exposure to 1 mg/L of Dinitramine for 12 h and Withdrawal in Herbicide-Free Water

	Withdrawal time, days			
Sample (N = 3-5)	0	1	3	7
Mean weight of the fish, g	297	259	215	212
Plasma				
Dinitramine	3.21[a] ±0.95	0.94 ±0.16	0.43 ±0.20	0.20 ±0.06
Metabolite III	ND[b]	ND	ND	ND
Metabolite V	ND	ND	ND	ND
Muscle				
Dinitramine	6.18 ±2.06	3.72 ±0.84	3.88 ±1.84	1.58 ±0.61
Metabolite III	ND	ND	ND	ND
Metabolite V	ND	ND	ND	ND
Bile				
Dinitramine	5.43 ±2.31	4.62 ±0.46	2.79 ±0.56	0.36 ±0.02
Metabolite III	0.60 ±0.03	0.61 ±0.12	0.46 ±0.07	Tr
Metabolite V	Tr[b]	4.35 ±1.44	3.87 ±1.42	2.11 ±0.97

[a] $\bar{X}$ ± SD. [b] ND is less than 0.01 mg/kg or mg/L; trace indicates the presence of a small peak with retention time corresponding to the standard.

RESULTS AND DISCUSSION

The concentration of dinitramine in blood plasma, muscle, and gallbladder bile of carp exceeded the exposure concentration of 1 mg/L after a 12-h period (Table II). After the carp were placed in fresh flowing water, samples at 0, 1, 3, and 7 days postexposure indicated rapid declines in concentrations of the herbicide in all three types of samples.

Dinitramine concentrations in plasma were approximately three times (3.21 mg/L) the bath concentration immediately after the exposure (Table II). However, the concentration had dropped to less than 30% the 0-day level by 1 day after exposure and to less than 7% (0.20 mg/L) by 7 days. Olson et al. (1975) reported a 79 and a 66% reduction in residues of the herbicide in carp and channel catfish plasma, respectively, after 24 h in fresh water. We found a 70% reduction in herbicide residues in plasma in this study. Thus, carp and channel catfish rapidly eliminated the herbicide dinitramine from the plasma after a single exposure.

Immediately after exposure the dinitramine concentration in carp muscle was 6.18 mg/kg (Table II). The concentrations had declined about 40% after 1 day in herbicide free water and 74% after 7 days to only 1.58 mg/kg. Kennedy et al. (1975) reported that whole body residues in bluegill sunfish tissue were eliminated rapidly following termination of a 30-day exposure to 1 ppm dinitramine. In our study, elimination of dinitramine was slower in fish muscle than in plasma and bile. However, since 74% of the herbicide was eliminated from the muscle in 7 days, the herbicide was probably being eliminated from the whole fish quite rapidly.

Gallbladder bile contained 5.43 mg/L of dinitramine immediately after the 12-h exposure, but by 7 days postexposure the concentration had declined to less than 7% (0.36 mg/L) of the 0-day value (Table II). However, the occurrence of measurable quantities of dinitramine metabolites in the bile indicated that the herbicide was biotransformed in the liver. Metabolites were not detected in the plasma or muscle.

Shimura and Sekizawa (1975) have shown N-hexuronyl conjugation on the amine of 2-amino-4-phenylthiazole.

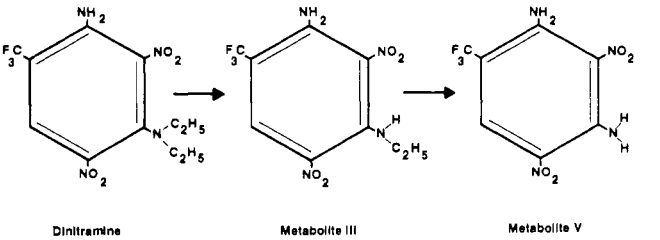


Figure 1. Metabolism of dinitramine in carp.

Dinitramine does contain a free amino group which could be conjugated; however, our methods do not extract water soluble conjugates of the herbicide.

The metabolism of dinitramine by microorganisms occurs via two pathways (Laanio et al., 1973; Smith et al., 1973). One pathway involved formation of a benzimidazole compound (metabolite II) and the other deethylation of dinitramine (metabolite III and V) as shown in Table I. DeWaide (1971), who studied the metabolizing capacity of fish tissue preparations in vitro, considered three reactions to be representative for biotransformation of xenobiotics: oxidative N-dealkylation, aromatic hydroxylation, and glucuronidation. He suggested that the enzymatic capacity measured in vitro represents to a certain degree the capacity in vivo and presented data to show that the liver is the most active tissue involved in biotransformation. Our study with carp documents the in vivo N-dealkylation of dinitramine by the liver.

N-Dealkylation of the herbicide by carp was indicated by the presence of metabolite III (monodeethylated) and metabolite V (dideethylated) in the bile. Dinitramine and metabolite V were confirmed in the bile by GC/MS. Metabolite III was not present in sufficient quantity for mass spectral analysis. However, III was identified by using two columns of different polarity in the GC, and the retention time on each column was identical with that of the standard. We could not detect the benzimidazole biotransformation product in this study.

Immediately after the exposure (0 day), bile contained 0.60 mg/L of metabolite III, but only a trace of metabolite V was present (Table II). After 1 day of elimination, the residue level of III was about the same as at 0 day and then gradually declined to a trace after 7 days. However, V was present at concentrations as high as dinitramine at 1 day. Although the concentration of V declined with time, almost six times as much of metabolite V as dinitramine remained after 7 days withdrawal. The presence of both III and V suggests stepwise N-deethylation (Figure 1). The absence of detectable amounts of these two metabolites in plasma but their presence in bile indicates that dinitramine was biotransformed in the liver and secreted into the bile (Table II).

CONCLUSIONS

(1) Residues of dinitramine in plasma, muscle, and gallbladder bile of carp declined during 7 days of withdrawal to 6, 25, and 7%, respectively, of the concentration found immediately after exposure. (2) A route of biotransformation of dinitramine in carp was shown to be N-dealkylation. (3) The appearance of metabolites III and V in gallbladder bile, but not in plasma or muscle, indicated that dinitramine was dealkylated in the liver.

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Sulfamethazine Residue in Calf Tissues

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Sulfamethazine prolonged release bolus was orally administered to calves in a single therapeutic dose of 225 mg per lb of body weight. Sulfamethazine residues in edible tissues were measured. The average biological half-life of the drug in various tissues fell within a narrow range of 0.87 to 1.05 days. Tissue residues in muscle, fat, liver, and kidney fell to control levels by 16 days. Data are presented which validate the method used in the range of 100–10 000 ppb. Tissue residue levels are not adversely affected if the tissues are frozen for up to 40 days prior to analysis.

In the development of sulfamethazine prolonged release bolus (Hava-span, Bayvet Corporation, Kansas City, Mo.) it was necessary to determine the clearance of the drug in four edible tissues—liver, kidney, muscle, and fat. Messersmith et al. (1967), administered sulfamethazine in combination with antibiotics to swine over a 14-week period. They found that the residue of sulfamethazine in edible tissues was less than 0.1 ppm 7 days after treatment was discontinued. Righter et al. (1971) administered sulfamethazine as a drench at a dose of 99 mg per kg per day and reported that sulfamethazine levels in calf tissues were reduced to 0.1 ppm by the eighth day after withdrawal of the drug.

Of the several methods (Fellig and Westheimer, 1968; Mooney and Pasarela, 1964; Righter et al., 1972) available, the procedure of Tishler et al. (1968) was chosen. The method described below is a modification of this procedure. Method validation data at both high and low levels of sulfamethazine are presented.

MATERIALS AND METHODS

Reagents and Chemicals. Sulfamethazine, USP, was obtained from B. L. Lemke and Company. All other reagents and solvents used were either analytical reagents or of the highest available purity.

Glassware. All glassware used in this study was washed with 6 N hydrochloric acid, 20% methanolic potassium hydroxide, detergent, distilled water, and acetone, in that order.

Experimental Design. Five groups of two steers and two heifers weighing between 400 and 600 lb were orally administered a single clinical dose (1 bolus/100 lb) of sulfamethazine prolonged release bolus. One group of calves was sacrificed at each of the following intervals: 2, 5, 10, 16, and 21 days. In addition, one steer and one heifer weighing 600 lb each were sacrificed and their liver, kidney, muscle, and fat collected and analyzed to determine sulfamethazine residues in control animals. All animals were kept on concrete floor and on a sulfa-free diet for 60 days prior to dosing and thereafter until sacrificed.

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Five pounds of adipose tissue was taken from the greater omentum and the large deposits on the body walls surrounding the kidneys. Both kidneys were collected and freed of fat and connective tissue. The entire liver was collected. Five pounds of striated muscle was taken from the neck region of the carcass and the fat removed.

The kidney and liver were each cut into 2- to 5-g pieces. The fat and muscle were each cut into chunks and ground in an electric grinder. Each tissue was then mixed thoroughly and sampled randomly. The tissue samples were analyzed immediately as described below. Weighed portions of the tissue samples were stored at −20 °C for studying the effect of freezing on sulfamethazine levels.

Analytical Methods. (a) *Liver, Kidney, or Muscle.* Tissue was weighed into a VirTis blender jar (200 mL) and homogenized with 90 mL of a mixture of acetone–chloroform (1:1). The extract was decanted through a glass wool plug into a 1-L round-bottomed flask. The extraction was repeated twice, each time decanting the extract through the glass wool plug into the same flask. The VirTis jar and the funnel were washed with several portions of the solvent mixture and the washes were collected in the round-bottomed flask. The solvent was removed from the combined extracts on a rotary evaporator at 70 °C. The residue was dissolved in 50 mL of hexane and transferred quantitatively to a 250-mL separatory funnel by washing with two 25-mL portions of hexane, two 3-mL portions of acetone, and two 25-mL portions of hexane, in that order. The organic phase was extracted into 10 mL of 1 N HCl, by vigorous shaking for 1 min, and the acid phase was drawn off into a 50-mL graduated cylinder. The organic phase was extracted three more times with 4-mL portions of 1 N HCl and then discarded. The acid phases were combined and diluted to 30 mL (in a graduated cylinder) with 1 N HCl and filtered through a 30-mL fine sintered glass funnel into a 125-mL Erlenmeyer flask. The graduated cylinder was washed with 2 mL of 1 N HCl and the washings filtered through a sintered glass funnel into the Erlenmeyer flask. Sulfamethazine content of the acid extract was determined by Bratton Marshall reaction.

(b) *Fat.* The fat was weighed into a 500-mL Erlenmeyer flask and to it was added 350 mL of acetone–chloroform (1:1). The mixture was stirred with a magnetic stirrer until